Root associated fungi on *Chimaphila umbellata* in south-eastern Norway

Master of Science Thesis

Line Holen

Department of Bioscience
University of Oslo

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Photo: Klaus Høiland

Forord

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Abstract

According to the Norwegian Red List, the evergreen plant species *Chimaphila umbellata* is considered endangered in Norway. Earlier reports have indicated that *C. umbellata* has a mixotrophic nutritional mode, and live in symbiotic relationships with both ericoid- (ERM) and ectomycorrhizal (ECM) fungi. Root samples from 12 *C. umbellata* shoots were collected from three localities, and 415 root tips were characterised using ITS sequencing. Instead of extracting DNA from each root tip, a recently developed direct PCR approach was tested. The results indicate that multiple colonisation of fungi is a common occurrence in *C. umbellata* root tips. 11.6 % of the samples clearly showed multiple PCR products after gel electrophoresis, and as much as 70.9 % of the sequences turned out as mixed sequence chromatograms. Only a fragment of the fungal symbionts in the root tips (13.5 %) could be identified. This indicate that direct PCR approach for non-ectomycorrhizal species are be less than ideal, possible due to the lack of fungal mantle on the root tips. High throughput sequencing is probably a much better option for assessing fungal species diversity associated with *C. umbellata*. No evidence of specificity to certain fungal lineages was found in this project. The majority of the successfully characterised sequences belonged to the Basidiomycota. The most abundant of the 31 OTUs belong to the genera *Cortinarius* and *Piloderma*, which are considered ectomycorrhizal species. Several fungal species normally associated with ericoid plants (e.g. *Meliniomyces* and *Oidiodendron*) were also detected in *C. umbellata* roots, as well as tentatively saprotrophic taxa such as *Mycena*. Hence, fungi with a wide array of nutritional modes and fungal lineages were detected in the root systems. The ECM fungi are especially interesting as they might form common mycelial networks (CMNs) with co-occurring pine trees. Though the extent of mixotrophy in adult plants remain unclear, CMNs could facilitate transport of carbon from trees to *C. umbellata*. In order to preserve this red-listed plant, it could therefore be necessary to preserve surrounding pine trees as well.

Key words: *Chimaphila umbellata*, *Pinus sylvestris*, mycorrhiza, direct PCR

Introduction

Fungi are a major part of soil microbial communities, where they function as decomposers, mycorrhizal mutualists, and pathogens. Mycorrhizal relationship is one of the most ancient and prevalent symbiosis of multicellular organisms known, and the majority of plants today form mycorrhizal relationship (Smith & Read 2008). The host plant benefits from increased effective surface area in the soil, which leads to more efficient root uptake of water and nutrients, while the fungi obtain carbohydrates from the host plant (Smith & Read 2008). Many of the most important interactions and functions of terrestrial ecosystems take place below ground, including carbon and nitrogen sequestration (Clemmensen et al. 2013). It is therefore important to obtain a better knowledge about mycorrhizal fungal diversity as they are key players in soil ecology (Dahlberg 2001).

Ectomycorrhiza (ECM) usually occur in ecosystems with low recycling of organic matter, and low nutrient availability (Zinger et al. 2011). ECM fungi are important players in the boreal forest due to their association with perennial woody plants, such as the families Pinaceae and Betulaceae. ECM fungi are phylogenetically diverse, involving a high number of species within Basidiomycota and Ascomycota, and a few Zygomycota (Dahlberg 2001). Some ECM associated fungi are also capable of forming other types of mycorrhiza, such as ericoid mycorrhiza (ERM), orchid mycorrhiza and arbutoid mycorrhiza (Bidartondo et al. 2004). The vegetative mycelia of ectomycorrhizal fungi have been shown to form networks of hyphal interconnections between plants, both intraspecific and interspecific, which allow transport of carbon from one plant to another (Finlay & Read 1986). It is believed that such common mycelial networks (CMNs) are crucial for mycoheterotrophic plant species (Bidartondo et al. 2004; Simard & Durall 2004).

Mycoheterotrophic plants are parasites, which obtain carbon either from saprotrophic fungi or indirectly from the surrounding plants via shared mycorrhizal fungi. Fully mycoheterotrophic plants depends totally on their fungal partners for carbon, as they no longer have photosynthesis (Leake 1994). The term mixotroph, or partially mycoheterotroph, is used about all plants that receive carbon from two sources. This include both plants that are close to full mycoheterotrophy, but still have some photosynthesis, and plants that are almost full autotrophs (Selosse & Roy 2009). Most mixotrophic plants are understorey plants, and it is

believed that mixotrophy evolved as a response to poor light conditions (Selosse & Roy 2009).

The tribe Pyroleae, in the plant family Ericaceae, mostly consists of mixotrophic species (Hynson & Bruns 2009; Tedersoo et al. 2007). Previous studies indicate that Pyroleae associates with endophytic, ectomycorrhizal and ericoid fungal species, which belong both to Ascomycota and Basidiomycota (Hynson & Bruns 2009; Tedersoo et al. 2007; Massicotte et al. 2008). Pyroleae species have been observed with arbutoid mycorrhiza, which share characteristics from both ericoid- and ectomycorrhiza (Leake 1994; Massicotte et al. 2008).

Fungal CMNs between plants have also been suggested as an important part of germination and growth of seedlings (Johansson & Eriksson 2013; Hynson et al. 2013; Simard & Durall 2004; Finlay & Read 1986). All Pyroleae species have dust seeds, which are characterised by lack of endosperm, and they are therefore usually very small and light. Since they lack endosperm, external carbon is needed in order for the seed to germinate, and this can be obtained by mycoheterotrophy (Johansson & Eriksson 2013; Leake 1994). Germination of seeds in Pyroleae species often occurs close to adult plants, thus raising the question of fungal host preference within this group (Johansson & Eriksson 2013). So far studies show a lack of fungal specificity in Pyroleae species (Hynson et al. 2013; Hynson & Bruns 2009; Tedersoo et al. 2007). However, much is still unknown about the nature of the relationship between mixotrophic plants and mycorrhizal fungi.

Chimaphila umbellata (L.) Barton is an evergreen low shrub in the Pyroleae tribe. It is only found in 89 localities in Norway, and considered endangered (EN) on the Norwegian red-list (Kålås et al. 2010). It only grows in the lowland and can be found in open, light abundant coniferous forests, usually dominated by pine trees (*Pinus sylvestris* L.). The soil is characteristic by its high content of sand, which makes the habitat well drained, and nutrient poor. *C. umbellata* forms underground root stems (rhizomes), from which asexual reproduction by clonal growth take place (Zobel & Antos 1987). One colony of *C. umbellata* can be considered as one individual that is connected underground.

A recent study found typical features of arbutoid mycorrhiza in *C. umbellata* (Massicotte et al. 2008). Some parts of the roots were observed sheathed in a thin mantle, usually also with a Hartig net between the root cells. The epidermal root cells are sometimes enlarged and

penetrated by hyphae which form coils underneath the cell wall without penetrating the cell membrane (Leake 1994; Massicotte et al. 2008).

Knowledge of a species ecology and biology is important in order to prevent local and national extinction. The most important fungal partners seem to be ECM fungi typically found in the roots of woody plants (Tedersoo et al. 2007; Massicotte et al. 2008). These fungi are believed to form a link between *C. umbellata* and the surrounding trees (Selosse & Roy 2009), and knowledge of which fungal partners it prefers could be crucial to preserve the species. Growth, density and genotype of host plants can explain the great diversity and patchy distribution of ECM fungi (Blaalid et al. 2012; Korkama et al. 2006; Zinger et al. 2011). Any disturbance to surrounding trees might have devastating consequences for belowground ECM species and therefore indirectly also for *C. umbellata*.

Lack of suitable microhabitats could be an explanation for why *C. umbellata* is so rare (Johansson & Eriksson 2013). A study on ECM fungi discover that almost all species was replaced after 50 cm (Tedersoo et al. 2003), and over 200 fungal species was found to associated with a single tree (Bahram et al. 2011). Since the diversity of mycorrhizal fungi is highly variable even on small scales, finding suitable microhabitats for the dust seeds could be difficult.

Given the fungal kingdom's age and genetic diversity, it is unlikely that a single-marker DNA barcode system will be capable of identifying every specimen or culture to species level (Schoch et al. 2012). For species identification in fungal kingdom it is most common to use the multi-copy nuclear ribosomal internal spacer (ITS) region (Schoch et al. 2012). This region have been established as a barcode for fungal species identification as it show both intra- and interspecies variability (Schoch et al. 2012). This region can also be amplifies from low quantity samples, such as plant roots and soil (Nilsson et al. 2008), which makes it a valuable barcode for examination of root associated fungi (RAF).

Normally, DNA would be extracted from a sample in order to run a PCR. This extraction is labour intensive, and involves a risk of contamination from airborne fungal spores. A recently developed approach can skip the DNA extraction part, and instead directly amplify DNA fragments from biological material (Shokralla et al. 2010). This approach have been used with success on ECM root tips (Velmala et al. 2013), and was adopted in the current study.

In this project, I will focus on identification of fungal symbionts present in the root tips of *C. umbellata*. I will analyse individual root tips to see if any specificity in regards to fungal lineages can be found, or whether root associated fungi include several lineages from typical ECM, ERM and saprotrophic fungi. I will also determine whether direct PCR and Sanger sequencing of individual root tips is a suitable method for identifying fungal species associated with *C. umbellata*.

Material and methods

Site descriptions

Samples were obtained on 31. May 2013 from three separate locations. At Hvervenmoen (N60° 8’29.60”, E10° 15’16.45”) in Buskerud County, there were only one colony of *C. umbellata* plants. At Prestmoen (N60°7'54.29", E10°12'39.60"), also in Buskerud County, there were approximately 25 colonies of *C. umbellata*. At the last locality, Bergermoen (N60°13'34.79", E10°21'16.84") in Oppland County, we found two colonies of *C. umbellata*.

The forest was dominated by mature pine trees (*Pinus sylvestris*), with a few spruce trees (*Picea abies*) and young oak trees (*Quercus robur*). Other understory plant species found at the three locations include *Vaccinium myrtillus, V. vitis-idaea, Avenella flexuosa, Convallaria majalis, Goodyera repens, Pyrola chlorantha, Monotropa hypopitys, Diphasiastrum complanatum* ssp. *complanatum* and *Orthilia secunda*. The mosses *Pleurozium schreberi* and *Hylocomium splendens* dominated the forest floor. Of fungi, we observed fruit bodies of *Russula*, *Piloderma* and *Elapomyces* close to *C. umbellata*.

Sampling methods

Two root samples from each of six colonies of *C. umbellata* plants were collected, adding up to 12 root samples. The length of the sampled root stems were between 10-30 cm, depending on the amount of thin side roots on the stem. Each sample were stored in a zip-lock bag and placed in a cooler for transportation. Root samples 1 and 2 were collected from the single *C. umbellata* colony at Hvervenmoen. Root samples 3 through 10 were sampled from four distinctly separate colonies at Prestmoen. The last root samples, 11 and 12, were taken from the largest colony at Prestmoen. We chose to only sample from one colony, as the smallest were deemed vulnerable to disturbance.

In order to avoid damaging this year’s recruitment only sterile shoots of *C. umbellata* were sampled. We also did not sample adjacent to flowering shoots, in order to avoid damage to shared roots, as the underground root stems usually connect several shoots.

Dissection and preservation

Each root sample was washed with distilled water in order to remove soil and other debris. Tweezers were used under dissection microscope in order to clean the thin root threads and root tips. Afterwards each root sample was rinsed three times with mqH_2O and placed on sterile paper to drain excessive water. Then each root stem was cut into smaller pieces and placed in petri dishes. Pieces of root stem only, without any thin side roots and root tips, were discarded. The petri dishes were stored in a freezer at -18 °C.

The original plan was to sample 35 root tips at random from each root sample. Since some of the root samples had less root tips on the thin side roots than we had estimated, we decided to instead sample 70 root tips from each colony as these could be seen as one individual due to clonal growth. Thus, the number of root tips from each root sample varied from 19 to 50, while the overall number of samples was stable at 69-70 for each colony. Scalpel and tweezers were used under a dissection microscope to sample each root tip. Each of the root tips sampled was placed into an Eppendorf tube containing 100 µl mqH_2O and stored in the -18 °C freezer. Pictures of the root tips were obtained by use of microscope with integrated camera equipment.

Direct PCR and Sanger sequencing

Thermo Scientific® Phire Plant Direct PCR kit was used to amplify the ITS region. The fungal specific primer pair ITS1-F and ITS4 (White et al. 1990) were used to avoid amplification of plant DNA and DNA from other organisms. In order to prepare the samples for PCR amplification, the root tips were first crushed with Qiagen® Tungsten Carbide beads (3mm) for 2 x 2 min, 20 rounds per second, and then the crushed material was transferred to new Eppendorf tubes. Instead of using the manufacturer's instruction, which is optimized for plant amplification, we modified the kit as followed: For each reaction 3.6 µl mqH2O, 10.0 µl 2X Plant PCR buffer, 2 µl ITS1-F, 2 µl ITS4, 0.4 µl Phire Hot Start II DNA Polymerase and 2.0 µl of the DNA template. PCR cycles were modified to denaturation at 98 °C for 5 min, cycling 98 °C for 25 s, 53°C for 30 s and 72 °C for 2 min in a total of 40 times, and final extension at 70 °C for 10 min.

In order to verify positive amplification before Sanger sequencing, the samples were run on a 1 % agarose gel with 70 V for 45 min. The positive samples were cleansed using Illustra ExoStar™. Cycle sequencing was performed using the ABI BigDye Terminator sequencing buffer and v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA). Sequences were processed on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems).

Bioinformatics analysis

All sequence chromatograms were inspected manually and proofread in Geneious version 7.1.3 created by Biomatters. Failed sequences and mixed sequence chromatograms were discarded. BLAST-search (Altschul et al. 1990) on the accepted sequences against both the NCBI and UNITE databases (Kõljalg et al. 2013) were used for taxonomic annotation of the sequences.

All sequences were clustered into operational taxonomic units (OTUs) using the tool BLASTclust, with 97 % sequence identity and 60 % sequence coverage as thresholds, implemented at the Bioinformatics Toolkit platform developed by Max-Planck Institute for Developmental Biology (Biegert et al. 2006).

Results

Observation of thin side roots and root tips

The sampled roots of *C. umbellata* consisted of a long root stem, connecting the plant clones in the colony, with various amounts of thin side roots. The thin side roots were branching into root tips. Figure 1 shows microscopy pictures of two thin side roots, one with swelling outer cells tentatively including hyphal coils, and the other with dark septated hyphae. No indications of a fungal mantle were observed, though dark septated hyphae forming Hartig net could be seen.

Figure 1. Fungal infection on *C. umbellata* root tips. a) Dark septated hyphae b) swollen epidermal cells with tentative fungal coils (arrowhead).

PCR amplification and sequencing

In total 415 root tips were sampled and prepared for PCR and sequencing as shown in table 1. We also prepared the samples for 454 sequencing, but these results have not arrived and will therefore have to be analysed in another study.

Table 1 Overview of 415 root tip samples taken from *C. umbellata*

	Samples	Percentage
Not sequenced after PCR amplification:	**202**	**48.5 %**
• Negative (no band)	154	37,1 %
• Smears (continuous band)	59	14,2 %
• Multiple products (several bands)	48	11,6 %
Sequenced (judged as single PCR product):	**213**	**51,3 %**
• Negative (NNNN)	6	1,4 %
• Mixed sequence chromatogram	151	36,4 %
• **Accepted**	**56**	**13,5 %**

After gel electrophoresis, we tried initial sequencing without separating single and multiple PCR products (1 % agarose gel, with 80 V for 30 min). It became clear that these largely resulted in unreadable mixed sequence chromatograms, and all later PCR amplification were run as described in the method section (1 % agarose gel with 70 V for 45 min). By running the samples longer on the gel, it became possible to separate single and multiple PCR products. However, even this modification could not catch all the samples with multiple PCR products, as shown by the large number of mixed sequence chromatograms (table 1). Hence, a large proportion of the sequences (70.9 %), which was derived from samples judged to have a single band on the gel, included mixed signals.

The accepted sequences (table 1) were all found to be unique ITS sequences, and are shown in supplementary table S1 along with highest BLAST-match from NCBI. These sequences were clustered into 31 OTUs (Table 2) when using 97% sequence similarity during the single linkage clustering analyses (BLASTclust). The higher-level taxonomic distribution of the OTUs is shown in Table 3. Agaricales and Atheliales were found to be the two most species rich groups.

Table 2. Overview of the 31 OTUs found in the root system of *C. umbellata*.

# OTU ID	Freq.	Reads	Query coverage	Max Identity	Accession	Description
8	1	13	100 %	99 %	DQ367911	*Cortinarius caperatus*
1	2	9	99 %	99 %	JQ711930	*Piloderma sphaerosporum*
15	2	3	100 %	99 %	JN882306	*Oidiodendron maius*
18	1	2	98 %	100 %	EU266681	*Cortinarius coleoptera*
26	1	2	100 %	98 %	DQ469291	*Piloderma olivaceum*
10	2	2	99 %	99 %	GQ159898	*Cortinarius laetissimus*
16	1	1	99 %	97 %	AY394885	*Meliniomyces bicolor*
14	1	1	97 %	97 %	KF002778	*Russula* sp.
2	1	1	100 %	99 %	HM240534	*Mycena galopus*
9	1	1	100 %	91 %	DQ367911	*Cortinarius caperatus*
27	1	1	100 %	99 %	FN669196	*Elaphomyces* sp
4	1	1	100 %	97 %	JQ711875	*Piloderma sphaerosporum*
13	1	1	100 %	99 %	JQ711935	*Piloderma* sp.
29	1	1	98 %	86 %	FN669196	*Elaphomyces* sp
22	1	1	99 %	83 %	JQ711930	*Piloderma* sp.
6	1	1	99 %	95 %	JQ711930	*Piloderma sphaerosporum*
11	1	1	99 %	96 %	JX975909	*Mortierella gemmifera*
28	1	1	99 %	96 %	DQ469281	*Piloderma byssinum*
25	1	1	98 %	96 %	DQ469281	*Piloderma byssinum*
24	1	1	100 %	98 %	DQ469291	*Piloderma olivaceum*
5	1	1	99 %	88 %	JQ711930	*Piloderma sphaerosporum*
19	1	1	99 %	95 %	EU266681	*Cortinarius coleoptera*
31	1	1	98 %	93 %	AY669678	*Cortinarius flexipes* var. *flabellus*
7	1	1	98 %	95 %	AY669673	*Cortinarius laetissimus*
30	1	1	99 %	91 %	HQ207028	*Thelebolales* sp.
3	1	1	100 %	99 %	KF850368	*Cadophora finlandica*
23	1	1	99 %	97 %	JN882306	*Oidiodendron maius*
21	1	1	100 %	95 %	JN882306	*Oidiodendron maius*
17	1	1	99 %	81 %	DQ384588	*Mycena* sp.
20	1	1	99 %	86 %	DQ384588	*Mycena* sp.
12	1	1	99 %	98 %	EF093178	*Meliniomyces variabilis*

Table 3. Overview of taxonomic affiliation of the total number of reads and the total number of OTUs.

Taxonomic affinity	Number of OTUs	Number of reads	% of OTUs	% of reads
Basidiomycota	**21**	**44**	**67,7**	**78,6**
• Agaricales	10	24	32,3	42,9
• Atheliales	10	19	32,3	33,9
• Russulales	1	1	3,2	1,8
Ascomycota	**9**	**11**	**29,0**	**19,6**
• (Incertae sedis) - Genus Oidiodendron	3	5	9,7	8,9
• Helotiales	3	3	9,7	5,4
• Eurotiales	2	2	6,5	3,6
• Thelebolales	1	1	3,2	1,8
Zygomycota	**1**	**1**	**3,2**	**1,8**

In general, the OTUs obtained high BLAST matches to GenBank accessions, with 74.2% of the OTUs having ≥ 95% match (Table 2). As we can read from table 2, only three of the OTUs were found in more than a single root sample. OTU#1 was found both at Hvervenmoen and Prestmoen, OTU#15 was found at two separate colonies at Prestmoen, and OTU#10 was found both at Prestmoen and Bergermoen.

Discussion

In this study, a direct PCR approach was chosen to amplify and sequence the ITS region of root associated fungi in the root system of *C. umbellata*. Direct PCR and Sanger sequencing of individual ectomycorrhizal root tips have previously been used, with great success, to characterize ECM fungi associated with *Picea abies* (Velmala et al. 2013). As far as we know, this is the first time this approach was used on a non-ECM plant.

However, it turned out that only a fraction, 13.5 % (table 1), of the root tips could be characterized using this approach. The first challenge was the discovery that 37.1 % (table 1) of our samples did not yield any ITS amplicons. Humic acid in soil can interfere with PCR amplification, and contamination by soil on the roots could perhaps explain some of the smears and negative amplifications (Yeates et al. 1998). There is also the possibility that the root tips were not colonised by fungi, or that the fungi present in the roots were not amplified by the selected primer pair (Bellemain et al. 2010). This has long been a problem within e.g. orchids, where the most commonly encountered fungal symbionts, species of *Tulasnella*, have poor match with the most common primer sets (Taylor & McCormick 2008). *Tulasnella* have also been found associated with *C. umbellata* and other Pyrolea species (Vincenot et al. 2008). It is therefore possible that some of the negative amplification could contain *Tulasnella* or other species with poor match with ITS1-F and ITS4.

The second challenge was the amount of multiple ITS bands amplified. We could identify 11.6 % (table 1) of the multiple PCR products by multiple bands on the gel. However, there were obviously many more, since 72.7 % of the sequences gave mixed signal chromatograms. The reason so many of the PCR products were difficult to interpret correctly by gel electrophoresis (i.e. whether a single or multiple band were present), could be due to similar lengths of DNA fragments or very weak signals on the gel. Another very labour intensive method, namely cloning each of the samples with multiple PCR products, have been used previously to solve this problem in a study with several *Pyrolae* species (Tedersoo et al. 2007).

The amount of sequences with mixed signal was higher than expected in comparison with a previous study, which found a mean of 25.6 % multiple PCR products on several *Pyrolae* species (Tedersoo et al. 2007). They observed that 75 % of the cloned multiple PCR products

from *Pyrolae* species represented endophytic fungal species (Tedersoo et al. 2007). As we can read from table 1, 48 % of the root tips either resulted in multiple bands (11.6 %) or mixed signals chromatograms (36.4 %) in this study. Dark septated hyphae could be observed within a root tip (figure 1), and the presence of several endophytes within the root tip sample would give multiple PCR products. The size of the root tip fragment that were used in the amplification process, would have a great impact on the amount of PCR products since larger samples would have a greater chance of multiple colonisation. Making sure that the root samples taken all are roughly the same size and small rather than large could increase the rate of single PCR products.

It is possible that the presence of a fungal mantle on ECM root tips reduces the amount of multiple PCR products. The ECM fungus could dominate over other fungi present in the root sample by sheer biomass, and therefore explain the good result by direct PCR on ECM species such as *P. abies* (Velmala et al. 2013). Previous studies indicate that ECM associated fungal species provide single PCR products also when colonizing root tips in *Pyrolae* species (Tedersoo et al. 2007). While no obvious fungal mantle were observed in *C. umbellata*, the presence of Hartig net and/or a thin mantle could explain why ECM associated fungi represent the majority of the detected species in this study.

In spite of the method problems, I was able to obtain 56 usable ITS sequences. As shown in table 3, it turned out that the majority of the OTUs belong to Basidiomycota (67.7 %). Several of these OTUs were from well-known ECM genera like *Cortinarius* and *Piloderma*. *Piloderma* and *Cortinarius* species are associated with acidic and nitrogen limited forests soil (Lilleskov et al. 2002). There are some indications that species within *Cortinarius* and *Piloderma* can produce enzymes that degrade soil organic matter, and from this process acquire nitrogen (Bödeker et al. 2014). This is particularly interesting when considering the fungally derived nitrogen previously found in *C. umbellata* (Tedersoo et al. 2007; Hynson et al. 2012).

Piloderma sphaerosporum have also been found to thrive in dry habitats and decline with increased rainfall (Jarvis et al. 2013). *C. umbellata* were found in localities that are dry due to sandy soil, so there is a possibility that *Piloderma* could be connected to drought tolerance in *C. umbellata*. *Piloderma* and *Cortinarius* also form ECM with pine trees (Lilleskov et al. 2002; Rosling et al. 2003) and are both described as medium-distance exploration types

(Agerer 2001). Hence, they could tentatively form CMNs, and possibly transfer carbon to *C. umbellata* from surrounding trees.

In addition, three OTUs with affinity to the genus *Mycena* were detected. This is intriguing as *Mycena* are believed to be saprotrophic species (Ghosh et al. 2003). However, in orchids some *Mycena* species are capable of forming specialized mycorrhiza with saprotrophic species. One study found that the mycoheterotrophic orchid *Gastrodia confusa* receives carbon from several different saprotrophic *Mycena* species (Ogura-Tsujita et al. 2009). It is possible that *C. umbellata* could have some sort of mycorrhiza with *Mycena*. However, its presence in the root system could also be due to decomposition activity of dead or dying root tips.

Several groups of ascomycete fungi were also detected, such as the genus *Meliniomyces* in the order Helotiales, which are commonly found in ericoid plants (Hambleton & Sigler 2005). Fungi within the order Helotiales have also been found as endophytes in association with ectomycorrhizal plant roots (Tedersoo et al. 2009). In this study we found the Helotiales fungi *Cadophora finlandica*, which have been found as an ECM fungi associated with *Pinus* (Hambleton & Sigler 2005). We also detected *Oidiodendron maius*, which have been found to form ericoid mycorrhiza with a wide range of ericoid plants (Hambleton & Currah 1997).

Noteworthy, the basidiomycete genus *Tricholoma* were not found in this study, but have previously been found associated with *C. umbellata* roots (Tedersoo et al. 2007). This study included relatively few samples, and these were randomly sampled from the roots taken at three separate localities. Since most of these were not sequenced and only one of four of these sequences were readable, we can definitely claim that we have not found all of the fungal species associated with *C. umbellata*.

Fruit bodies of the ECM ascomycete *Elaphomyces* was observed twice at the Prestmoen locality. Interestingly, this fungus was also detected in the root samples. *Elaphomyces* have underground fruiting bodies, and rely on animals to dig it up and disperse its spores. Exposed sites due to animal disturbance could allow dust seeds from *C. umbellata* to come into direct contact with existing CMNs.

The fact that several ECM fungi were detected on the roots of *C. umbellata* in this study indicate that it receives nutrients from surrounding trees through the CMN. Several studies have noted that *C. umbellata* receives fungal-derived nitrogen, which likely enables it to grow in nutrient poor pine forests (Zimmer et al. 2007; Tedersoo et al. 2007). However, a recent study could not find any indication that *C. umbellata* receives carbon from its fungal partners and it is therefore believed that the mature plants are primarily autotrophic (Hynson et al. 2012). Since it grows in areas with abundant light, mature plants likely acquire carbon from photosynthesis, thought there are indications that *C. umbellata* could receive carbon from fungal partners when light availability is low (Tedersoo et al. 2007). Other studies indicate that the degree of mycoheterotrophy might also depend on the environment in which the plant grows, the season, and development stage (Hynson et al. 2009; Tedersoo et al. 2007).

Regardless of the degree of mixotrophy found in mature *C. umbellata* plants, the dust seeds are initially mycoheterotrophic and thus depends on external carbon in order to develop (Johansson & Eriksson 2013). Germination of *C. umbellata* seeds could be spatially restricted to locations adjacent to plant roots that are already supporting ectomycorrhizal fungi (Johansson & Eriksson 2013), which have been suggested for orchid germination (Bidartondo et al. 2004). There are also some indications that *C. umbellata* could be more specific concerning fungal hosts during germination (Johansson & Eriksson 2013). If so, a very interesting question is whether the fungal partners during germination are retained in adult plants.

Clearcut logging might reduce recruitment in *C. umbellata* if the dust seeds depend on existing CMN with mature trees in order to germinate. Clearcut logging has also been found to cause a shift in ECM key species, such as *Piloderma*, which have been found to decrease drastically when mature pine trees are removed (Jones et al. 2003). Since we have found several ECM species associated with *C. umbellata*, including several species of *Piloderma*, it is reasonable to assume that *C. umbellata* would be affected by any disturbance to surrounding pine trees. Even if the adult plants of *C. umbellata* is primarily autotrophic, it could still be dependent on CMN between itself and surrounding trees with respect to nitrogen acquisition.

In order to prevent local and national extinction, both the existing populations of *C. umbellata* and the co-occurring trees should therefore be conserved. In order to preserve the population

of *C. umbellata*, destruction or alteration of its habitat by clearcutting or urban development should be avoided. It should also be noted that any long time nitrogen addition to the system is likely to change the fungal composition (Lilleskov et al. 2002), and fertilizing should also be avoided in areas close to *C. umbellata* for this reason. Moreover, *C. umbellata* show preference for light-abundant areas which means that some active management in order to prevent the populations from being overshadowed by spruce saplings or other sources might be preferable in order to prevent population decline.

Conclusion

Direct PCR and Sanger sequencing proved not to be ideal for detection of root associated fungi in *C. umbellata*. A high number of the samples were not amplified by the PCR reaction and there were a high number of multiple PCR products. While it might be possible to reduce the number of multiple PCR product by reducing the size of the root samples, I believe that further studies would benefit more from choosing to use high throughput sequencing to analyse root associated fungi.

When examining the limited number of sequences, this study could find no indications of specificity to certain fungal lineages. On the contrary, we found several different lineages of both ECM and ERM fungi. *C. umbellata* form mycorrhiza with ECM fungi from both Basidiomycota and Ascomycota, notably the basidiomycete genera *Cortinarius* and *Piloderma*. These are previously known for ECM association with coniferous trees, which could indicate that *C. umbellata* is dependent on the surrounding trees though CMNs. Future studies should address the mechanisms behind seed germination in *C. umbellata,* and whether the fungal partners during germination matches the species that have been found associated with adult plants. It would also be very interesting to compare root associated fungi on C. umbellata with co-occurring plants, especially trees, to see if they actually share fungal symbionts.

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Appendix

Table S1. Overview of accepted sequences, along with their OUT#ID, score, coverage, identity and accession number, with BLAST-match from NCBI. The name indicate locality, samples beginning with the name 01 an2 02 are from Hvervenmoen, samples with the name 03 through 10 are from Prestmoen, and 11 plus 12 are from Bergermoen.

Name	OTU #ID	Total score	Query cover	Identity	Accession	Description
01_1	1	1099	99 %	99 %	JQ711930	*Piloderma sphaerosporum*
01_10	2	1158	100 %	99 %	HM240534	*Mycena galopus*
01_7	3	935	100 %	99 %	KF850368	*Cadophora finlandica*
02_1	11	992	99 %	96 %	JX975909	*Mortierella gemmifera*
02_16	12	660	99 %	98 %	EF093178	*Meliniomyces variabilis*
02_2	13	1101	100 %	99 %	JQ711935	*Piloderma* sp.
02_3	14	1125	97 %	97 %	KF002778	*Russula* sp.
03_10	15	918	97 %	98 %	JN882306	*Oidiodendron maius*
03_16	16	1419	99 %	97 %	AY394885	*Meliniomyces bicolor*
03_32	17	438	99 %	81 %	DQ384588	*Mycena* sp.
03_33	18	1061	98 %	100 %	EU266681	*Cortinarius coleoptera*
03_35	19	941	99 %	95 %	EU266681	*Cortinarius coleoptera*
03_36	18	1061	96 %	100 %	EU266681	*Cortinarius coleoptera*
03_38	20	527	99 %	86 %	DQ384588	*Mycena* sp.
05_18	21	848	100 %	95 %	JN882306	*Oidiodendron maius*
05_30	22	719	99 %	83 %	JQ711930	*Piloderma* sp.
05_5	23	876	99 %	97 %	JN882306	*Oidiodendron maius*
06_1	24	1029	100 %	98 %	DQ469291	*Piloderma olivaceum*
06_10	15	950	100 %	99 %	JN882306	*Oidiodendron maius*
06_11	15	950	100 %	99 %	JN882306	*Oidiodendron maius*
06_17	25	970	98 %	96 %	DQ469281	*Piloderma byssinum*
06_2	26	856	100 %	96 %	DQ469291	*Piloderma olivaceum*
06_24	27	1158	100 %	99 %	FN669196	*Elaphomyces* sp.
06_3	26	953	99 %	95 %	DQ469291	*Piloderma olivaceum*
06_30	28	976	99 %	96 %	DQ469281	*Piloderma byssinum*
08_13	29	778	98 %	86 %	FN669196	*Elaphomyces* sp.
08_29	30	717	99 %	91 %	HQ207028	*Thelebolales* sp.
09_18	31	863	98 %	93 %	AY669678	*Cortinarius flexipes* var. *flabellus*
09_32	10	1003	99 %	99 %	GQ159898	*Cortinarius laetissimus*
10_13	1	1086	99 %	99 %	JQ711930	*Piloderma sphaerosporum*
10_14	1	1083	99 %	99 %	JQ711930	*Piloderma sphaerosporum*
10_15	1	1092	99 %	99 %	JQ711930	*Piloderma sphaerosporum*
10_20	1	1086	99 %	99 %	JQ711875	*Piloderma sphaerosporum*
10_21	1	950	100 %	98 %	JQ711875	*Piloderma sphaerosporum*
10_26	1	1086	99 %	99 %	JQ711930	*Piloderma sphaerosporum*
10_27	4	1027	100 %	97 %	JQ711875	*Piloderma sphaerosporum*
10_28	1	1053	98 %	97 %	JQ711930	*Piloderma sphaerosporum*
10_5	1	1040	99 %	97 %	JQ711930	*Piloderma sphaerosporum*

10_7	5	785	99 %	88 %	JQ711930	*Piloderma* sp.
10_8	6	966	99 %	95 %	JQ711930	*Piloderma sphaerosporum*
11_2	7	852	98 %	95 %	GQ159898	*Cortinarius laetissimus*
12_13	8	1088	100 %	98 %	DQ367911	*Cortinarius caperatus*
12_14	8	1203	100 %	99 %	DQ367911	*Cortinarius caperatus*
12_18	8	1212	99 %	99 %	DQ367911	*Cortinarius caperatus*
12_22	8	1074	100 %	99 %	DQ367911	*Cortinarius caperatus*
12_23	8	1173	99 %	99 %	DQ367911	*Cortinarius caperatus*
12_24	8	1206	97 %	99 %	DQ367911	*Cortinarius caperatus*
12_27	8	1195	99 %	99 %	DQ367911	*Cortinarius caperatus*
12_28	9	953	100 %	91 %	DQ367911	*Cortinarius caperatus*
12_29	8	1171	99 %	98 %	DQ367911	*Cortinarius caperatus*
12_30	8	1190	99 %	99 %	DQ367911	*Cortinarius caperatus*
12_31	8	990	100 %	91 %	DQ367911	*Cortinarius caperatus*
12_32	8	1203	99 %	99 %	DQ367911	*Cortinarius caperatus*
12_5	8	1158	99 %	98 %	DQ367911	*Cortinarius caperatus*
12_6	10	929	98 %	96 %	GQ159898	*Cortinarius laetissimus*
12_7	8	1162	100 %	99 %	DQ367911	*Cortinarius caperatus*